

Detoxification effects and potential mechanisms of NP-induced hepatotoxicity by ethanolic extracts from *Clinacanthus nutans* (Burm.f.) Lindau leaf

Xueman Xiao

South China Agricultural University

Congying Chen

South China Agricultural University

Huiying Huang

South China Agricultural University

Jian Shi

South China Agricultural University

Huan Liu

South China Agricultural University

Chunhong Liu (✉ liuch@scau.edu.cn)

South China Agricultural University

Research Article

Keywords: Nonylphenol, *Clinacanthus nutans* (Burm.f.) Lindau, Flavonoids, Nrf2

Posted Date: January 5th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2430459/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Clinacanthus nutans (Burm.f.) Lindau (*C. nutans*) is native to tropical Asian countries and is an important ingredient in the traditional medicinal system. It is of interest because of the flavonoids it contains, which are rare in plants. It has traditionally been used to treat herpes infections and snake venoms. However, the ability of *C. nutans* to counteract exogenous chemical-induced hepatotoxicity is not yet known.

Nonylphenol (NP) is a widely distributed environmental endocrine disruptor, and the liver is one of the target organs for its toxic effects. In this study, we designed in vitro tests to investigate the protective effect of ethanolic extracts from *C. nutans* leaf (CE) on NP-induced hepatotoxicity and its molecular mechanism. Sixty μM NP was selected as the optimum to treat Hep G2 cells for 24 h to establish a cell injury model, and the intervention doses of CE were 0.25, 0.5, 1, 2 mg/mL, followed by ERK inhibitor (PD98059), P38 inhibitor (SB202190), Nrf2 inhibitor (ML385) to assist in validation of the intervention mechanism. The results showed that CE could reverse NP-induced Hep G2 cytotoxicity and upregulated cell survival, cellular GSH content, CAT viability and ATP content which was probably achieved by inhibiting ERK/P38 mitogen-activated protein kinases (MAPKs) pathway and activating the nuclear erythroid 2-related factor 2/Heme Oxygenase-1 (Nrf2/HO-1) pathway. These data suggest that CE play a detoxifying role in NP-induced hepatotoxicity, which may be achieved by inhibiting the ERK/P38 MAPKs pathway and activating the Nrf2/HO-1 pathway.

1. Introduction

NP is a recognized environmental endocrine disruptor and one of the 13 priority hazardous substances listed by the European Union [1]. NP is utilized for the chemical industry production of surfactants, detergents, emulsifiers, pesticides, lubricants, and oil additives. [2]. Humans can be exposed to NP in daily life through a variety of ways, such as inhalation of air, intake of contaminated water, skin contact with NP-containing consumer products, and consumption of NP-containing foods [3, 4]. Food intake has become the main route of human exposure to NP. In the 2007 Chinese Total Diet Study, Yumin Niu et al. measured the NP content in 144 food samples and found that NP was detected in 143[4]; 55 of 62 drinking water samples from 31 major cities in China were found the existence of NP [5]. Currently, most NP toxicity studies have focused on NP's immune and reproductive toxicity [6–8], while its hepatotoxicity has been less frequently reported. The liver is one of the essential organs in the body, not only for its metabolic functions but also for detoxification and the secretion and excretion of bile. Therefore, the health of the liver has a direct impact on the individual's health.

C. nutans, commonly known as the dual-use plant for medicine and food, is widely distributed in tropical areas, mainly in Southeast Asia, such as Thailand and Malaysia. In China, it is primarily distributed in Hainan, Jieyang, etc. Traditionally, *C. nutans* can treat herpes infections and snake bites [9], and research reported that *C. nutans* possesses analgesic, anti-inflammatory, and antiviral activities against Herpes simplex virus (HSV) and Varicella-zoster virus (VZV)[10]. In Thailand, the plant has been accepted as an essential herb for primary health care by the Thai Ministry of Public Health [11]. Extracts of *C. nutans* have been demonstrated in various studies to exhibit a variety of biological actions, including anti-

tumour, anti-inflammatory, antioxidant, nephrotoxic, and antiviral properties. [12–18]. The effectiveness of the medicine depends on the composition. Studies have reported that *C. nutans* leaves contain triterpenoids, phytosterols, chlorophyll derivatives, sulfur-containing glycosides, and phenolic and flavonoid compounds [11], phenolic and flavonoid compounds are of great interest. The main flavonoid compounds in *C. nutans* are shaftoside, isoorientin, orientin, isovitexin, and vitexin, which are rare in plants. These compounds possess important biological activities, including hepatoprotective activity (isoorientin), antimicrobial activity (isoorientin, vitexin), and antioxidant activity (isovitexin) [19]. In addition, some common polyphenolic compounds, such as catechins, gallic acid and chlorogenic acid, have been shown to have critical physiological activities [20]. The pharmacological effects of *C. nutans* have been noted in some studies, but no specific molecular mechanisms have been revealed, especially the hepatoprotective effects [9].

Nrf2 is a transcription factor. Under normal physiological conditions, Nrf2 binds to its repressor Kelch-like ECH binding protein 1 (Keap1) and is maintained at low levels in the cytosol; when cells are under oxidative stress, Nrf2 breaks away from the control of Keap1 and translocates to the nucleus, activating the transcription and expression of a series of antioxidant enzymes, phase II detoxification enzymes and phase III transport proteins in response to oxidative stress [21]. Nrf2 has been reported to be activated by MAPKs[22], a general term for a family of serine-threonine protein kinases. In response to extracellular stimuli, MARKs can transmit signals from the cell surface to the cell interior as a tertiary kinase cascade, thereby regulating cellular physiological processes such as inflammatory response and apoptosis [23, 24].

To observe the whole-food effect of *C. nutans* leaf, this study was designed to establish a liver injury model using NP-induced Human hepatocellular carcinoma cells (Hep G2) to investigate the detoxification effect of CE on NP-induced hepatotoxicity and the possible mechanism and to provide a theoretical basis for further research on NP toxicity and the development of CE health care functions.

2. Materials And Methods

2.1 Preparation of plant extracts

The name *Clinacanthus nutans* (Burm.f.) Lindau has been checked (<http://www.worldfloraonline.org/>). The *C. nutans* were collected from Hainan, China.

The leaves of *C. nutans* were purchased from the planting base of Songqing Technology Co. The fresh *C. nutans* leaves were freeze-dried, crushed and passed through a 60 mesh sieve; the powder was accurately weighed, and the extracts were extracted by ultrasonication (45°C, 90min, and 100W) by adding 70% ethanol by volume at a ratio of 1:15. The extracts were centrifuged at 3000 r/min for 10 min. The leaf residue of *C. nutans* was repeatedly extracted by ultrasonication. The extracts were filtered under a vacuum after centrifugation. The filtrate was removed from ethanol and concentrated at 40 ~ 50 °C by

rotary evaporation under reduced pressure to obtain the extracts; the extracts were freeze-dried to obtain CE, which was stored at -20 °C for use.

2.2 Determination of total phenols content (TPC) and total flavonoids content (TFC)

TPC in CE was determined using the Folin-Ciocalteu method.

Configuration of the standard curve: The master batch was diluted into 50, 100, 150, 200, 250, 300, 350, and 400 µg/mL of standard solution. Added 125 µL of standard solution to 125 µL of Folin-Ciocalteu's phenol reagent and let stand for 6 min. Added 7% Na₂CO₃ (1.25 mL), made up to 3 mL with water (1.5 mL with water) and left for 90 min. The absorbance was measured at 760 nm, and the standard curve was plotted with absorbance as the vertical coordinate and gallic acid concentration as the horizontal coordinate.

Determination of the sample: The CE was re-dissolved, and the absorbance was measured by 125 µL according to the above procedure. The regression equation of the standard curve calculated the total polyphenol content of CE.

TFC in CE was determined using the NaNO₂-Al (Cl)₃ method.

Preparation of standard curve: The mother liquor was diluted into 25, 50, 75, 100, 125, 150, 175, and 200 µg/mL of standard solution, respectively. Accurately pipetted 1 mL of rutin preparation solution into a 25 mL colorimetric tube, added 5% NaNO₂ solution 0.4 mL and mixed well, left for 6 min, then added 10% Al(Cl)₃ solution 0.4 mL and mixed well, left for 6 min, finally added 4% NaOH solution 4.0 mL, added with 80% ethanol and fixed to the scale, left for 15 min at room temperature, measured the absorbance at 510 nm. The standard curve was plotted with absorbance as the vertical coordinate and rutin concentration as the horizontal coordinate.

Determination of the sample: re-dissolved CE, took 1 mL into a 25 mL colorimetric tube, added 5% NaNO₂ solution 0.4 mL and shaken well, left for 6 min, then added 10% Al (Cl)₃ solution 0.4 mL and shaken well, left for 6 min, finally added 4% NaOH solution 4.0 mL, added 80% ethanol to the scale, left for 15 min at room temperature, measured the absorbance at 510 nm. The absorbance at 510 nm was measured, and the content of flavonoids in CE was calculated by substituting it into the standard curve.

2.3 Cell culture

Hep G2 ((hepatocellular carcinoma; Cat. No. CL-0103) was obtained from Procell Life Sciences Co., Ltd., Wuhan, China. The cell culture medium was MEM complete culture medium containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PEN-Streptomycin: 10000 U/ml) and 1% L-glutamine. The cell culture environment was 37°C and 5% CO₂, and the culture medium was changed every 1–2 days under normal conditions. NP and dimethyl sulfoxide (DMSO) were purchased from Shanghai Macklin Biochemical Co., Ltd and MP Biomedicals LLC, USA, respectively. According to the experimental design,

log phase cells were taken and treated with dimethyl sulfoxide (solvent control, final concentration < 0.1%), NP (40, 50, 60, and 70 μ M) or NP (60 μ M) combined with CE (0.25, 0.5, 1, 2 mg/mL) treatment. Cell samples were subsequently obtained for correlation analysis.

2.4 Cell viability

Cells in the logarithmic growth phase were inoculated into 96-well plates at 3000 cells/well. 24 h later, different concentrations of NP or CE were added, and six parallels were set for each concentration. After incubation for 24 h, ten μ L of CCK-8 reagent (Beijing Solaibao Technology Co., Ltd.) was added to each well. The absorbance was measured at 450 nm after a two-hour dark incubation.

2.5 Determination of cellular oxidative stress-related indicators

The glutathione (GSH) content kit and catalase (CAT) activity assay kit were purchased from Nanjing Jiancheng Bioengineering Institute. After NP or CE treatment, the cells were washed with pre-chilled PBS, lysed by adding lysis solution (Shanghai Beyotime Biotechnology Ltd.) for 30 min, collected in centrifuge tubes, centrifuged for 10 min and then the supernatant was taken for the corresponding assay according to the instructions. GSH content was expressed as micromoles per gram of protein, and CAT activity was expressed as units of enzyme activity per mg of protein.

2.6 Determination of ATP content

The ATP content assay kit was purchased from Shanghai Beyotime Biotechnology Ltd. After 24 h of NP or CE treatment, ATP lysis solution was added, and the cells were lysed on ice for 30 min.

2.7 Western blot analysis

Cells were inoculated in 6-well plates for 24 h and then treated with NP or CE for 24 h. Cells were subsequently lysed with RIPA lysis solution on ice for 30 min, and the supernatant was centrifuged to determine their protein concentration. Protein samples were boiled in a boiling water bath for 7 min and stored in aliquots at -80°C ambient. 15 μ g of protein samples were added to each well, and protein electrophoresis was performed in 10% SDS-PAGE, then transferred onto PVDF membranes and blocked in 5% skim milk powder for 1.5 h. Subsequently, protein samples were incubated with p-ERK, ERK, p-P38, P38, p-JNK, JNK, HO-1, Nrf2 and NQO1 primary antibodies overnight at 4°C. Horseradish peroxidase-labelled secondary antibody (Affinity) was added and incubated at room temperature at a ratio of 1:6000 for one h. The optical density values of the samples were analyzed by Image J. The results were expressed as the optical density value of the target protein divided by the optical density value of the internal reference protein.

2.8 Statistical analysis

All raw data were collated in a Microsoft Excel database, while statistical analysis was performed using SPSS 18.0. Data results were expressed as Mean $\pm$ SD. One-way ANOVA was used to analyze differences

between groups, and significant differences were accepted at $P < 0.05$, and highly significant differences were accepted at $P < 0.01$ confidence level.

3. Results

3.1 TPC and TFC of CE

As shown in Fig. 1 (d) and (e), the standard curve equation of gallic acid was $y = 0.0025x - 0.0699$, $R^2 = 0.9941$, and the standard curve equation of rutin was $y = 0.0016x - 0.027$, $R^2 = 0.9996$. The TPC in CE was 57.37mg/g and the TFC was 41.56 mg/g.

3.2 Effect of NP administration on the viability of Hep G2 cells

As shown in Fig. 2(a), after using different concentrations of NP (3.125, 6.25, 12.5, 25, 50, 100, 200 μM) on Hep G2 cells for 24 h, cell viability was not significantly different compared with the control group at concentrations of 3.125 ~ 50 μM ; cell viability was reduced to 13.33% and 2.04% at concentrations of 100 μM and 200 μM , with highly significant differences compared with the control group ($P < 0.01$). To select the appropriate NP concentration, Hep G2 cells were treated with 40, 50, 60, 70, 80, 90 and 100 μM for 24 h. The results are shown in Fig. 2(b) at concentrations of 40 μM and 50 μM was not significantly different compared with the control group; at concentrations of 60 μM and 70 μM , cell viability decreased to 87.89% and 55.03%, respectively, with highly significant differences compared with the control group ($P < 0.01$). Subsequent experiments were selected at 0, 40, 50, 60 and 70 μM .

To investigate whether NP produced cytotoxicity to Hep G2 cells, GSH content and CAT activity, which are indicators of oxidative stress, and ATP content, which is an indicator of mitochondrial function, were measured. As shown in Fig. 2(c)(d), when NP was applied to Hep G2 cells for 24 h, GSH content showed a dose-dependent decrease, and the decrease was statistically significant at 50, 60 and 70 μM ($P < 0.01$). Meanwhile, CAT vitality showed a trend of the first decrease and then increased, and the vitality dropped to the lowest at 60 μM ($P < 0.01$), but there was no significant difference between the CAT vitality of 60 μM and 70 μM ($P > 0.05$). As shown in Fig. 2(e), when NP was applied to Hep G2 cells for 24 h, the ATP content showed a tendency to increase and then decrease, and the decrease was statistically significant at 60 μM and 70 μM compared with the control group ($P < 0.05$, $P < 0.01$).

3.3 Effect of NP administration on Nrf2 pathway in Hep G2 cells

To investigate whether NP affects the classical antioxidant and detoxification Nrf2 pathway, Hep G2 cells were treated with different concentrations of NP for 24 h. As shown in Fig. 3(a)(b)(c), compared with the control group, the protein contents of Nrf2, HO-1 and NQO1 decreased in a dose-dependent manner; Nrf2

protein content decreased significantly at 40, 50, 60 and 70 μ M ($P < 0.01$), and down-regulated its downstream proteins HO-1 and NQO1 protein content. The results indicated that NP affects the expression of Nrf2 pathway-related proteins.

3.4 Effect of NP administration on MAPKs pathway in Hep G2 cells

The Nrf2 pathway may be activated by its upstream MAPKs pathway in addition to being directly activated by ROS. To explore whether NP affects the MAPKs pathway, Hep G2 cells were treated with different concentrations of NP for 24 h. As shown in Fig. 3(d)(e)(f), The expressions of p-ERK and p-P38 appeared to increase and then decrease while the total expressions of ERK and total P38 gradually decreased with increasing doses of NP. p-ERK/ERK ratio showed a gradual increase, and p-P38/P38 showed an increase and then a decrease. This indicated that NP activated p-ERK and p-P38 at 40–60 μ M, and at 70 μ M, NP inhibits p-ERK and p-P38 by suppressing total ERK and total P38; while p-JNK/JNK is activated only at 70 μ M ($P < 0.01$). The following experiments used the NP concentration with 60 μ M.

3.5 Intervention effect of CE on NP-induced cytotoxicity

To determine the concentration range of CE intervention in Hep G2, Hep G2 cells were treated with different concentrations of CE (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 mg/mL) for 24 h. The results of CCK-8 experiments showed that CE at low concentrations (0.125, 0.25, 0.5, 1, 2, 4, 8, 16 mg/mL) had an activating effect on Hep G2 cell viability ($P < 0.05$) at low concentration (0.125 mg/mL), while an inhibitory dose-dependent impact ($P < 0.01$) was observed at high concentrations (4–16 mg/mL).

The results of CCK-8 experiments showed a reduction in cell viability in the NP-treated group ($P < 0.01$), indicating that the NP cell injury model was successfully constructed; cell viability was increased after the intervention with different concentrations (0.25 to 2 mg/mL) compared with the NP-treated group ($P < 0.01$). Therefore, 0.25, 0.5, 1 and 2 mg/mL were selected as CE intervention concentrations in the subsequent experiments.

To investigate whether CE improved Hep G2 cytotoxicity caused by NP, GSH content, CAT activity and ATP content were analyzed; as shown in Fig. 4, GSH content was downregulated in the NP group compared with the control group ($P < 0.05$), while GSH content was upregulated after CE intervention ($P < 0.05$, $P < 0.01$). Meanwhile, CAT viability decreased in the NP group compared with the control group ($P < 0.01$) and increased after CE intervention with 1 mg/mL and 2 mg/mL ($P < 0.01$); furthermore, ATP content decreased in the NP group compared with the control group ($P < 0.01$), and increased in a dose-dependent manner after CE intervention ($P < 0.01$). It was suggested that CE's intervention effect was on NP-induced cytotoxicity.

3.6 CE alleviated the cytotoxicity of NP via the Nrf2 pathway

To further study the protective mechanism of CE on NP-treated Hep G2 cells, the expression of Nrf2-related pathway proteins was assayed. Nrf2 and HO-1 were down-regulated in the NP group compared with the control group ($P < 0.05$, $P < 0.01$). At the same time, the content of Nrf2 increased and then decreased after CE intervention, and the expression of its downstream pathway protein HO-1 was upregulated. This suggested that CE intervention regulates the expression of Nrf2-related pathway proteins in NP-treated Hep G2 cells.

3.7 CE alleviated the cytotoxicity of NP via the ERK1/2 pathway and P38

To investigate whether CE affected the expressions of MAPKs pathway proteins which were the upstream regulators of Nrf2, CE was applied to NP-treated Hep G2 cells for 24 h. As shown in Fig. 5, CE at 0.25-1 mg/mL dose-dependently down-regulated the activations of p-ERK/ERK and p-P38/P38 by NP, while NP combined with 2 mg/mL CE upregulated the relative expression of p-ERK/ERK, p-P38/P38 proteins, suggesting that CE at 0.25 ~ 1 mg/mL had a downregulatory effect on NP-induced MAPKs activation, and therefore, 1 mg/mL was chosen for the following experiments.

3.8 CE affected Nrf2 pathway through ERK1/2 and P38 to alleviate the cytotoxicity of NP

Next, we verified the effect of CE on the activation of p-ERK/ERK due to NP. ERK1/2 is an essential upstream signaling molecule in the Nrf2/HO-1 pathway. As shown in Fig. 6(a), NP group had an activating effect on p-ERK/ERK, while CE intervention significantly decreased the expression of p-ERK/ERK and upregulated the expression of downstream pathway proteins Nrf2 and HO-1. Meanwhile, ERK1/2 inhibitor (PD98059) down-regulated p-ERK/ERK expression and upregulated downstream pathway protein Nrf2 and HO-1 expression in NP-treated Hep G2 cells. These results were consistent with the results after CE intervention in NP-treated Hep G2 cells, suggesting that CE might upregulate the expression of transcription factor Nrf2 and its downstream detoxification protein HO-1 by inhibiting NP-induced p-ERK/ERK expression in Hep G2 cells.

To verify the effect of CE on NP-induced activation of p-P38/P38, the P38 inhibitor (SB202190) was used on NP-treated Hep G2 cells. p38 is the same as ERK1/2, which is an important upstream signaling molecule in the Nrf2/HO-1 pathway. As shown in Fig. 6(b), the NP group had an activating effect on p-P38/P38, while CE intervention significantly reduced the expression of p-P38/P38 and upregulated the expression of downstream pathway proteins Nrf2 and HO-1. Meanwhile, SB202190 down-regulated the expression of p-P38/P38 and upregulated the expression of downstream pathway proteins Nrf2 and HO-1 in NP-treated Hep G2 cells. These results were also consistent with the results after CE intervention in NP-treated Hep G2 cells, suggesting that CE might upregulate the expression of transcription factor Nrf2 and its downstream detoxification protein HO-1 by inhibiting NP-induced expression of p-P38/P38 in Hep G2 cells.

To verify whether CE regulated NP-induced Hep G2 cell injury via Nrf2, Hep G2 cells co-interacted with CE and NP were intervened using the Nrf2 inhibitor ML385. As shown in Fig. 6(c), the NP group downregulated the expression of Nrf2 and thus HO-1 compared to the control group, and the expression of Nrf2 and HO-1 increased after intervention with CE; further addition of the Nrf2 inhibitor (ML385) resulted in a dose-dependent downregulation of Nrf2 and HO-1 expression compared to the CE and NP co-interaction group; subsequently, it was explored whether Nrf2 inhibition affected the expression of upstream proteins ERK1/2, P38, and the results showed that NP group activated the expression of p-ERK/ERK, p-P38/P38, and the expression of p-ERK/ERK, p-P38/P38 was significantly reduced after the intervention of CE; meanwhile, the addition of Nrf2 inhibitor (ML385) dose-dependently upregulated the expression of p-ERK/ERK, p-P38/P38 compared with the CE and NP co-interaction group.

4. Discussion

NP is a recognized class of environmental toxicants; food intake has become the main route of human exposure to NP. There are abundant studies on NP's reproductive and developmental toxicity [6, 7, 25], but there are fewer studies on NP-induced liver damage. As the major metabolic and detoxification organ, the liver contributes to health maintenance, which is essential in increasing exposure to environmental pollutants. The present study found that different concentrations of NP caused dose-dependent damage to Hep G2 cells. NP resulted in decreased cell survival, decreased expression of oxidative stress-related indexes GSH content and CAT vitality, and decreased mitochondrial function-related indexes ATP content in Hep G2 cells. Similar conclusions were reached in previous studies on both cellular and animal experiments [26–28]. Dandan Shan et al. found that NP-treated Hep G2 cells caused a decrease in cell survival [26]; Rui Shi et al. found a decrease in GSH content and CAT viability in liver tissue of the NP-treated group in rats [27]; Lin-Lin Hu et al. showed that NP exposure in mice resulted in decreased ATP levels and abnormal mitochondrial function [28]. To explore the molecular mechanisms of NP toxicity in more detail, we investigated the Nrf2/HO-1 signaling pathway. The results showed that NP downregulated the expression of Nrf2, a key transcription factor for liver detoxification, and its downstream antioxidant proteins HO-1 and NQO1. This downregulation might be achieved by activating MAPKs-related pathway proteins p-ERK/ERK, p-P38/P38, and p-JNK/JNK. This is the same as the previous findings [29, 30]. Nrf2 play a key role in regulating the mRNAs of many phases I and II drug metabolism genes as well as some efflux transport proteins, such as HO-1, a downstream protein of Nrf2, whose increased expression is regulated by the activation of Nrf2 and is thought to have strong antioxidant and anti-inflammatory properties [31]. NP-induced cellular injury may be mediated by activation of the MAPKs pathway, and thus by inhibiting the level of Nrf2 down-regulates the expression of antioxidant proteins HO-1 and NQO1, thus imbalancing cellular oxidative stress levels.

Flavonoid compounds, as a class of plant secondary metabolites, are of wide interest for their biological activities. Studies have shown that flavonoid compounds have a high antioxidant capacity, control the accumulation of reactive oxygen species (ROS), and are highly relevant to human health [32]. In the present study, the TFC was 41.56 mg/g, and the TPC was 57.37 mg/g in CE. CE was used to intervene in NP-treated Hep G2 cells and was found to dose-dependently reverse the cellular damage caused by NP. At

the epistatic level, CE reversed the NP-induced decrease in cell survival, oxidative stress-related indicators of GSH content and CAT activity, and downregulation of mitochondrial function-related indicators of ATP; in mechanistic studies, CE partially reversed NP-induced activations of p-ERK/ERK and p-P38/P38 and upregulated the expression of Nrf2 and downstream detoxification protein HO-1. Previous studies showed that the ethanolic extracts from *C. nutans* leaf are rich in flavonoids and polyphenols, which are of interest because the flavonoids contained in them are rare in plants[33], C-glycosyl flavones, including schaftoside, isovitexin, vitexin, orientin, and isoorientin, were identified by analysis of *C. nutans* leaf extracts. Studies on various phytochemicals indicated that they possess important biological activities, including antimicrobial activity(isoorientin, vitexin), hepatoprotective activity (schaftoside, isoorientin) and antioxidant activity (isovitexin)[19, 34], as evidenced by their ability to upregulate the antioxidant stress pathway involving Nrf2 and HO-1 production [35]; related studies also showed that flavonoid compounds could regulate the transcription factor Nrf2 and its downstream protein HO-1 to improve the inflammatory response and oxidative stress [36–39], such as Nagla A El-Shitany et al. found that icariin could regulate carrageenan-induced acute inflammation through the HO-1 / Nrf2 signaling pathway [36]; Xinnong Liu et al. reported that isoliquiritigenin could improve acute pancreatitis in mice by inhibiting oxidative stress and regulating the Nrf2/ HO-1 pathway [37]. In addition, related studies further found that *C. nutans* leaf extracts down-regulates p-ERK, p-P38, and p-JNK activation in lipopolysaccharide (LPS)-induced RAW264.7 macrophage inflammation[40]. These findings suggested that the detoxifying effect of CE on NP-induced hepatotoxicity might be achieved through the inhibition of p-ERK/ERK and p-P38/P38 activation and, thus upregulation of Nrf2 expression levels and activation of the downstream antioxidant protein HO-1, which had an ameliorating effect on the imbalance of oxidative stress levels in cells.

To further verify whether CE affected Nrf2/HO-1 pathway mainly via ERK1/2 and P38 and thus alleviated NP-induced hepatotoxicity, cells were intervened using ERK inhibitor (PD98059), P38 inhibitor (SB202190) and Nrf2 inhibitor (ML385). The results showed that CE could exert the effects of ERK inhibitor and P38 inhibitor inhibited NP-induced p-ERK/ERK and p-P38/P38 activation [41] and further upregulated the expression of Nrf2 and downstream detoxification protein HO-1; meanwhile, the ameliorative effect of CE on NP-induced hepatotoxicity was diminished by using Nrf2 inhibitor, the expression of Nrf2 and downstream HO-1 was reduced, and the expressions of upstream p-ERK/ERK, p-P38/ P38 were further determined. Both were found to be activated again. The above results suggested that CE mainly affects the Nrf2/HO-1 pathway via ERK1/2 and P38 to alleviate the NP-induced cell injury.

In the current study, NP could cause dose-dependent cell damage to Hep G2, including decreased cell survival, reduced GSH content and CAT activity, which were indicators of oxidative stress evaluation, and reduced ATP, which was an indicator of mitochondrial function; this might be due to the activation of MAPKs pathway by NP and down-regulation of the expression of transcription factor Nrf2 and its downstream detoxification proteins HO-1 and NQO1. CE was used to intervene in NP-treated Hep G2 cells, and CE ameliorated NP-induced cell damage significantly. Further investigation of the mechanism of CE intervention showed that the intervention was mainly achieved by inhibiting the ERK/P38 MAPKs pathway and activating the Nrf2/HO-1 pathway. In light of the previous studies, it is proposed that the

hepatoprotective effect of *C. nutans* might be caused by its flavonoids. However, more in-depth investigations should be conducted to validate this. In a word, *C. nutans* will hopefully be developed as a novel healthcare product to combat exogenous chemical toxicity and protect the liver from damage.

Declarations

Ethical Approval and Consent to Participate

Hep G2 ((hepatocellular carcinoma; Cat. No. CL-0103), which was analyzed with authenticated STR locus and tested for mycoplasma contamination, was obtained from Procell Life Sciences Co., Ltd., Wuhan, China. This declaration is not applicable.

Consent for publication

This study uses cell lines and this declaration is not applicable.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Authors' contributions

C, Liu has planned and supervised the research work, and X, Xiao has executed the experimental research work under the supervision of C, Chen, H, Huang and H, Liu. X, Xiao finally structured the entire manuscript and drafted the manuscript. C, Liu and J, Shi have edited the manuscript. All the authors have read the manuscript and approved it for submission.

Funding

This work was supported by the Natural Science Foundation of Guangdong Province (No. 2021A1515010825), Guangdong Key R&D Program, Department of Science and Technology of Guangdong Province (No. 2019B020210002), Generic Technique Innovation Team Construction of Modern Agriculture of Guangdong Province (No. 2021KJ130) and Guangdong Provincial Key Laboratory of Food Quality and Safety (No. 2020B1212060059).

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

References

1. Lu Z, Gan J: **Analysis, toxicity, occurrence and biodegradation of nonylphenol isomers: a review.** *Environ Int* 2014, **73**:334-345.

2. Huang YF, Wang PW, Huang LW, Lai CH, Yang W, Wu KY, Lu CA, Chen HC, Chen ML: **Prenatal Nonylphenol and Bisphenol A Exposures and Inflammation Are Determinants of Oxidative/Nitrative Stress: A Taiwanese Cohort Study.** *Environ Sci Technol* 2017, **51**(11):6422-6429.
3. Chung SWC: **The development of isomer-specific analysis of branched 4-nonylphenol in food for dietary exposure - a critical review of analytical methods and occurrence in foodstuffs.** *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2021, **38**(5):842-855.
4. Lu D, Yu L, Li M, Zhai Q, Tian F, Chen W: **Behavioral disorders caused by nonylphenol and strategies for protection.** *Chemosphere* 2021, **275**:129973.
5. Fan Z, Hu J, An W, Yang M: **Detection and occurrence of chlorinated byproducts of bisphenol a, nonylphenol, and estrogens in drinking water of china: comparison to the parent compounds.** *Environ Sci Technol* 2013, **47**(19):10841-10850.
6. Chen ML, Lee HY, Chuang HY, Guo BR, Mao IF: **Association between nonylphenol exposure and development of secondary sexual characteristics.** *Chemosphere* 2009, **76**(7):927-931.
7. Ishibashi H, Hirano M, Matsumura N, Watanabe N, Takao Y, Arizono K: **Reproductive effects and bioconcentration of 4-nonylphenol in medaka fish (*Oryzias latipes*).** *Chemosphere* 2006, **65**(6):1019-1026.
8. Niu Y, Zhang J, Duan H, Wu Y, Shao B: **Bisphenol A and nonylphenol in foodstuffs: Chinese dietary exposure from the 2007 total diet study and infant health risk from formulas.** *Food Chem* 2015, **167**:320-325.
9. Zulkipli IN, Rajabalaya R, Idris A, Sulaiman NA, David SR: **Clinacanthus nutans: a review on ethnomedicinal uses, chemical constituents and pharmacological properties.** *Pharm Biol* 2017, **55**(1):1093-1113.
10. Kongkaew C, Chaiyakunapruk N: **Efficacy of Clinacanthus nutans extracts in patients with herpes infection: systematic review and meta-analysis of randomised clinical trials.** *Complement Ther Med* 2011, **19**(1):47-53.
11. Khoo LW, Audrey Kow S, Lee MT, Tan CP, Shaari K, Tham CL, Abas F: **A Comprehensive Review on Phytochemistry and Pharmacological Activities of Clinacanthus nutans (Burm.f.) Lindau.** *Evid Based Complement Alternat Med* 2018, **2018**:9276260.
12. Haron NH, Md Toha Z, Abas R, Hamdan MR, Azman N, Khairuddean M, Arsad H: **In Vitro Cytotoxic Activity of Clinacanthus nutans Leaf Extracts Against HeLa Cells.** *Asian Pac J Cancer Prev* 2019, **20**(2):601-609.
13. Kuo X, Herr DR, Ong WY: **Anti-inflammatory and Cytoprotective Effect of Clinacanthus nutans Leaf But Not Stem Extracts on 7-Ketocholesterol Induced Brain Endothelial Cell Injury.** *Neuromolecular Med* 2021, **23**(1):176-183.
14. Ismail NZ, Md Toha Z, Muhamad M, Nik Mohamed Kamal NNS, Mohamad Zain NN, Arsad H: **Antioxidant Effects, Antiproliferative Effects, and Molecular Docking of Clinacanthus nutans Leaf Extracts.** *Molecules* 2020, **25**(9).

15. Wu JS, Kao MH, Tsai HD, Cheung WM, Chen JJ, Ong WY, Sun GY, Lin TN: **Clinacanthus nutans Mitigates Neuronal Apoptosis and Ischemic Brain Damage Through Augmenting the C/EBPbeta-Driven PPAR-gamma Transcription.** *Mol Neurobiol* 2018, **55**(7):5425-5438.
16. Le CF, Kailaivasan TH, Chow SC, Abdullah Z, Ling SK, Fang CM: **Phytosterols isolated from Clinacanthus nutans induce immunosuppressive activity in murine cells.** *Int Immunopharmacol* 2017, **44**:203-210.
17. Azemi AK, Mokhtar SS, Rasool AHG: **Clinacanthus nutans Leaves Extract Reverts Endothelial Dysfunction in Type 2 Diabetes Rats by Improving Protein Expression of eNOS.** *Oxid Med Cell Longev* 2020, **2020**:7572892.
18. Mahmood, II, Ismail IS, Alitheen NB, Normi YM, Abas F, Khatib A, Rudiyanto, Latip J: **NMR and LCMS analytical platforms exhibited the nephroprotective effect of Clinacanthus nutans in cisplatin-induced nephrotoxicity in the in vitro condition.** *BMC Complement Med Ther* 2020, **20**(1):320.
19. Chelyn JL, Omar MH, Mohd Yousof NS, Ranggasamy R, Wasiman MI, Ismail Z: **Analysis of flavone C-glycosides in the leaves of Clinacanthus nutans (Burm. f.) Lindau by HPTLC and HPLC-UV/DAD.** *ScientificWorldJournal* 2014, **2014**:724267.
20. Khoo LW, Mediani A, Zolkeflee NKZ, Leong SW, Ismail IS, Khatib A, Shaari K, Abas F: **Phytochemical diversity of Clinacanthus nutans extracts and their bioactivity correlations elucidated by NMR based metabolomics.** *Phytochemistry Letters* 2015, **14**:123-133.
21. Qi Z, Ci X, Huang J, Liu Q, Yu Q, Zhou J, Deng X: **Asiatic acid enhances Nrf2 signaling to protect HepG2 cells from oxidative damage through Akt and ERK activation.** *Biomed Pharmacother* 2017, **88**:252-259.
22. Ma BX, Meng XS, Tong J, Ge LL, Zhou G, Wang YW: **Protective effects of Coptis chinensis inflorescence extract and linarin against carbon tetrachloride-induced damage in HepG2 cells through the MAPK/Keap1-Nrf2 pathway.** *Food & Function* 2018, **9**(4):2353-2361.
23. Zheng Z, Shi L, Sheng Y, Zhang J, Lu B, Ji L: **Chlorogenic acid suppresses monocrotaline-induced sinusoidal obstruction syndrome: The potential contribution of NFκB, Egr1, Nrf2, MAPKs and PI3K signals.** *Environmental Toxicology and Pharmacology* 2016, **46**:80-89.
24. Fan X, Jiang Y, Wang Y, Tan H, Zeng H, Wang Y, Chen P, Qu A, Gonzalez FJ, Huang M *et al*: **Wuzhi tablet (Schisandra Sphenanthera extract) protects against acetaminophen-induced hepatotoxicity by inhibition of CYP-mediated bioactivation and regulation of NRF2-ARE and p53/p21 pathways.** *Drug Metab Dispos* 2014, **42**(12):1982-1990.
25. Lin HC, Li HY, Wu YT, Tsai YL, Chuang CY, Lin CH, Chen WY: **Bayesian inference of nonylphenol exposure for assessing human dietary risk.** *Sci Total Environ* 2020, **713**:136710.
26. Shan D, Wang J, Di Q, Jiang Q, Xu Q: **Steatosis induced by nonylphenol in HepG2 cells and the intervention effect of curcumin.** *Food Funct* 2022, **13**(1):327-343.
27. Shi R, Liu Z, Liu T: **The antagonistic effect of bisphenol A and nonylphenol on liver and kidney injury in rats.** *Immunopharmacol Immunotoxicol* 2021, **43**(5):527-535.

28. Hu LL, Li HG, Li XM, Xu Y, Pang YQ, Wang B, Wang JL, Sun SC: **Nonylphenol exposure-induced oocyte quality deterioration could be reversed by melatonin supplementation in mice.** *Environ Pollut* 2022, **305**:119317.
29. Ke Q, Yang J, Liu H, Huang Z, Bu L, Jin D, Liu C: **Dose- and time-effects responses of Nonylphenol on oxidative stress in rat through the Keap1-Nrf2 signaling pathway.** *Ecotoxicol Environ Saf* 2021, **216**:112185.
30. Gu W, Wang Y, Qiu Z, Dong J, Wang Y, Chen J: **Maternal exposure to nonylphenol during pregnancy and lactation induces microglial cell activation and pro-inflammatory cytokine production in offspring hippocampus.** *Sci Total Environ* 2018, **634**:525-533.
31. Zeng J, Deng Z, Zou Y, Liu C, Fu H, Gu Y, Chang H: **Theaflavin alleviates oxidative injury and atherosclerosis progress via activating microRNA-24-mediated Nrf2/HO-1 signal.** *Phytother Res* 2021, **35**(6):3418-3427.
32. Dias MC, Pinto D, Silva AMS: **Plant Flavonoids: Chemical Characteristics and Biological Activity.** *Molecules* 2021, **26**(17).
33. Aliyu A, Shaari MR, Ahmad Sayuti NS, Reduan MFH, Sithambaram S, Noordin MM, Shaari K, Hamzah H: **Subacute Oral Administration of Clinacanthus nutans Ethanolic Leaf Extract Induced Liver and Kidney Toxicities in ICR Mice.** *Molecules* 2020, **25**(11).
34. Liu M, Zhang G, Song M, Wang J, Shen C, Chen Z, Huang X, Gao Y, Zhu C, Lin C *et al*: **Activation of Farnesoid X Receptor by Schaftoside Ameliorates Acetaminophen-Induced Hepatotoxicity by Modulating Oxidative Stress and Inflammation.** *Antioxid Redox Signal* 2020, **33**(2):87-116.
35. Ong WY, Herr DR, Sun GY, Lin TN: **Anti-Inflammatory Effects of Phytochemical Components of Clinacanthus nutans.** *Molecules* 2022, **27**(11).
36. El-Shitany NA, Eid BG: **Icariin modulates carrageenan-induced acute inflammation through HO-1/Nrf2 and NF- κ B signaling pathways.** *Biomed Pharmacother* 2019, **120**:109567.
37. Liu X, Zhu Q, Zhang M, Yin T, Xu R, Xiao W, Wu J, Deng B, Gao X, Gong W *et al*: **Isoliquiritigenin Ameliorates Acute Pancreatitis in Mice via Inhibition of Oxidative Stress and Modulation of the Nrf2/HO-1 Pathway.** *Oxid Med Cell Longev* 2018, **2018**:7161592.
38. Ma L, Wu F, Shao Q, Chen G, Xu L, Lu F: **Baicalin Alleviates Oxidative Stress and Inflammation in Diabetic Nephropathy via Nrf2 and MAPK Signaling Pathway.** *Drug Des Devel Ther* 2021, **15**:3207-3221.
39. Egbujor MC, Saha S, Buttari B, Profumo E, Saso L: **Activation of Nrf2 signaling pathway by natural and synthetic chalcones: a therapeutic road map for oxidative stress.** *Expert Rev Clin Pharmacol* 2021, **14**(4):465-480.
40. Mai CW, Yap KS, Kho MT, Ismail NH, Yusoff K, Shaari K, Chin SY, Lim ES: **Mechanisms Underlying the Anti-Inflammatory Effects of Clinacanthus nutans Lindau Extracts: Inhibition of Cytokine Production and Toll-Like Receptor-4 Activation.** *Front Pharmacol* 2016, **7**:7.
41. Li Z, You K, Li J, Wang Y, Xu H, Gao B, Wang J: **Madecassoside suppresses proliferation and invasiveness of HGF-induced human hepatocellular carcinoma cells via PKC-cMET-ERK1/2-COX-2-**

Figures

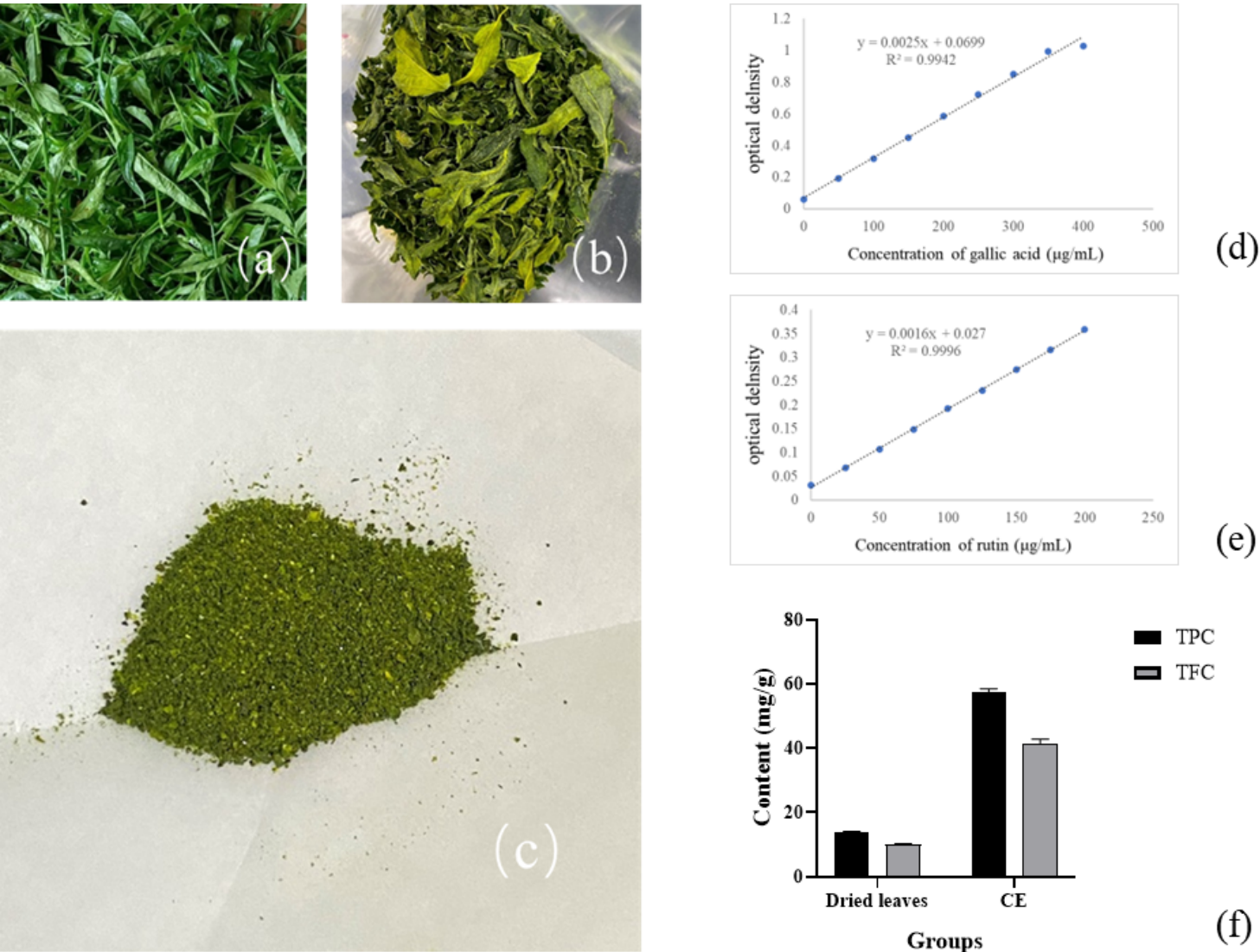


Figure 1

C. nutans leaves, CE, TPC and TFC (a) Fresh C. nutans leaves; (b) Freeze-dried C. nutans leaves; (c) CE; (d) Gallic acid standard curve; (e) Rutin standard curve; (f) TPC and TFC of CE (n=3).

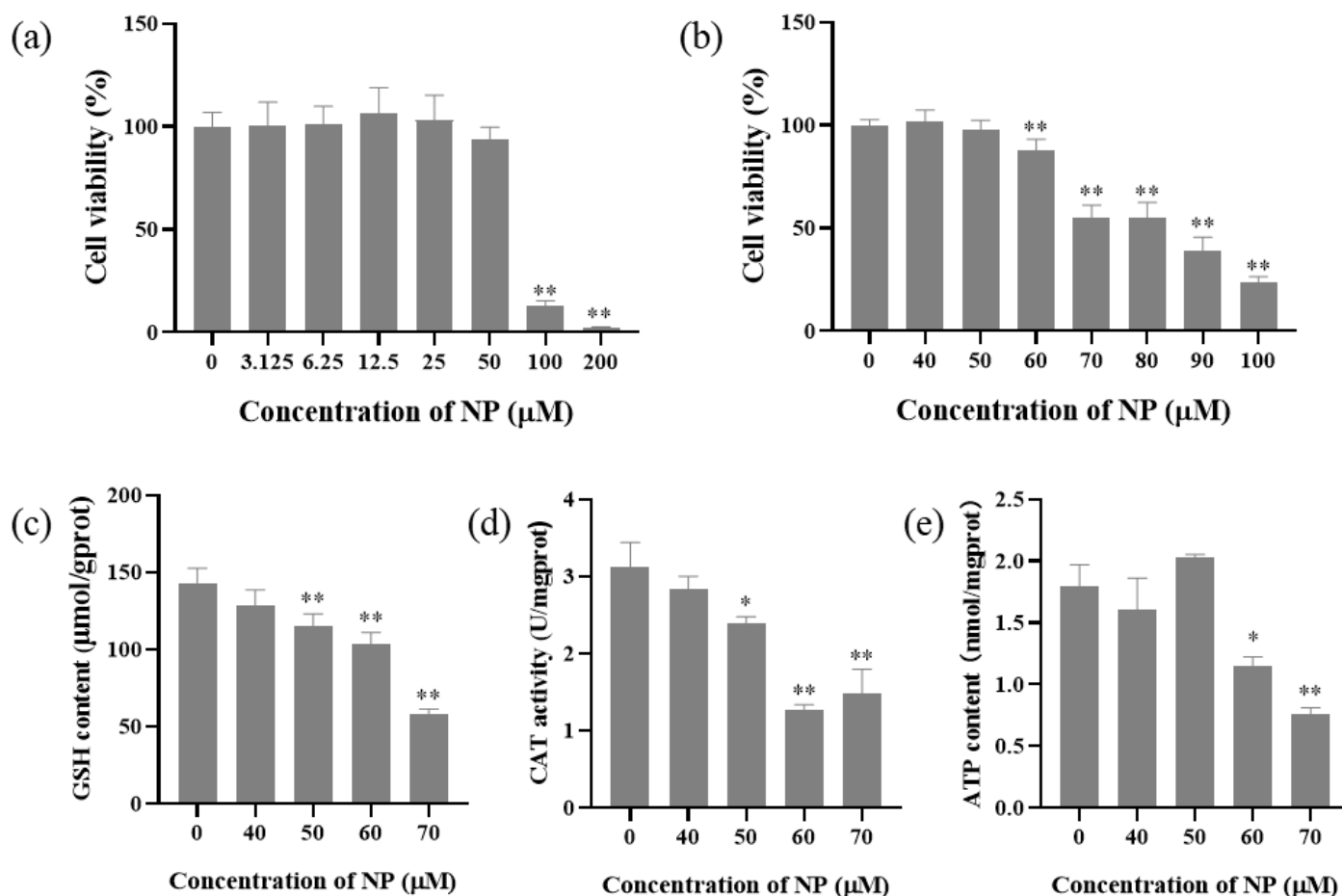


Figure 2

Cytotoxicity of NP on Hep G2 cells (a) (b) Cell viability of Hep G2 cells after 24h incubation with different concentrations of NP (n=6); (c) (d) (e) GSH content, CAT activity and ATP content of Hep G2 cells after 24h incubation with different concentrations of NP (n=3). (*: $P<0.05$ compared with the control group; **: $P<0.01$ compared with the control group.)

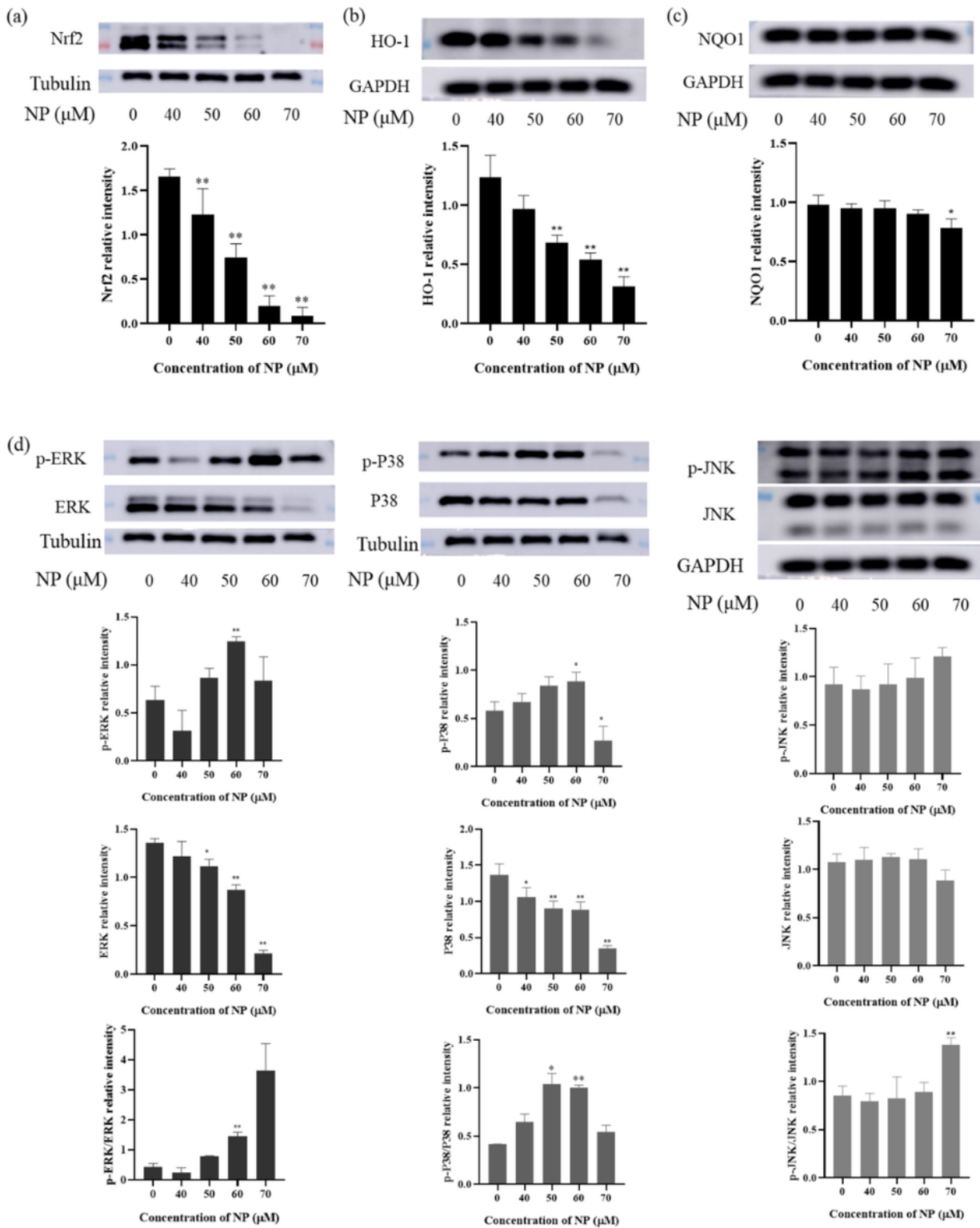


Figure 3

NP affects the MAPKs/Nrf2 pathway in Hep G2 cells (a) (b) (c) NP affects the Nrf2 pathway in Hep G2 cells (n=3); (d) (e) (f) NP affects the MAPKs pathway in Hep G2 cells (n=3). (*: $P < 0.05$ compared with the control group; **: $P < 0.01$ compared with the control group.)

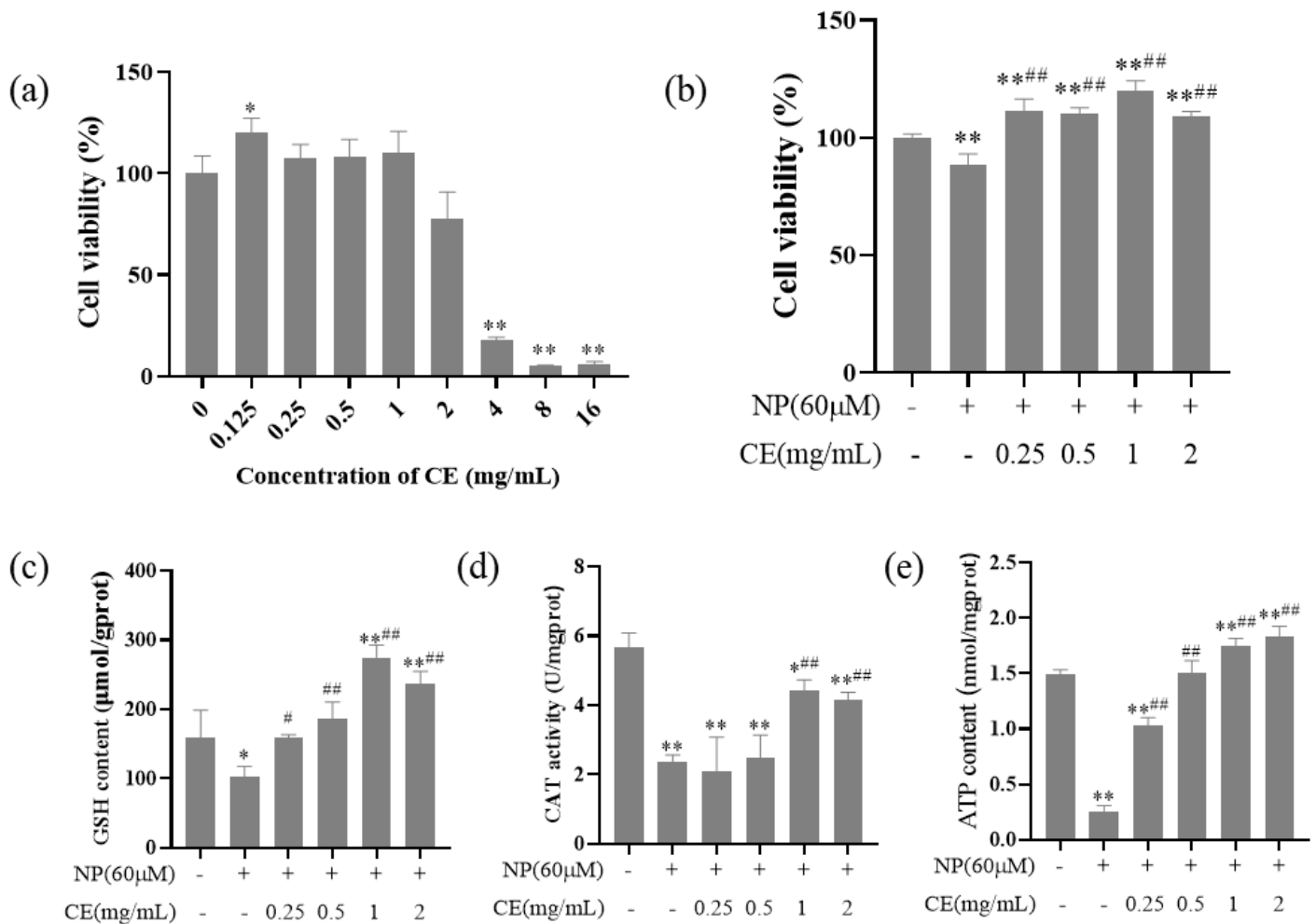


Figure 4

CE has an alleviating effect on NP-induced cytotoxicity (a) Cell viability of Hep G2 cells after 24h treatment with different concentrations of CE(n=6); (b) Cell viability of NP-treated Hep G2 cells after 24h intervention with different concentrations of CE (n=6); (c) (d) (e) GSH content, CAT activity and ATP content of NP-treated Hep G2 cells after 24h intervention with different concentrations of CE (n=3). (*: $P<0.05$ compared with the control group; **: $P<0.01$ compared with the control group; #: $P<0.05$ compared with the NP group; ##: $P<0.01$ compared with the NP group.)

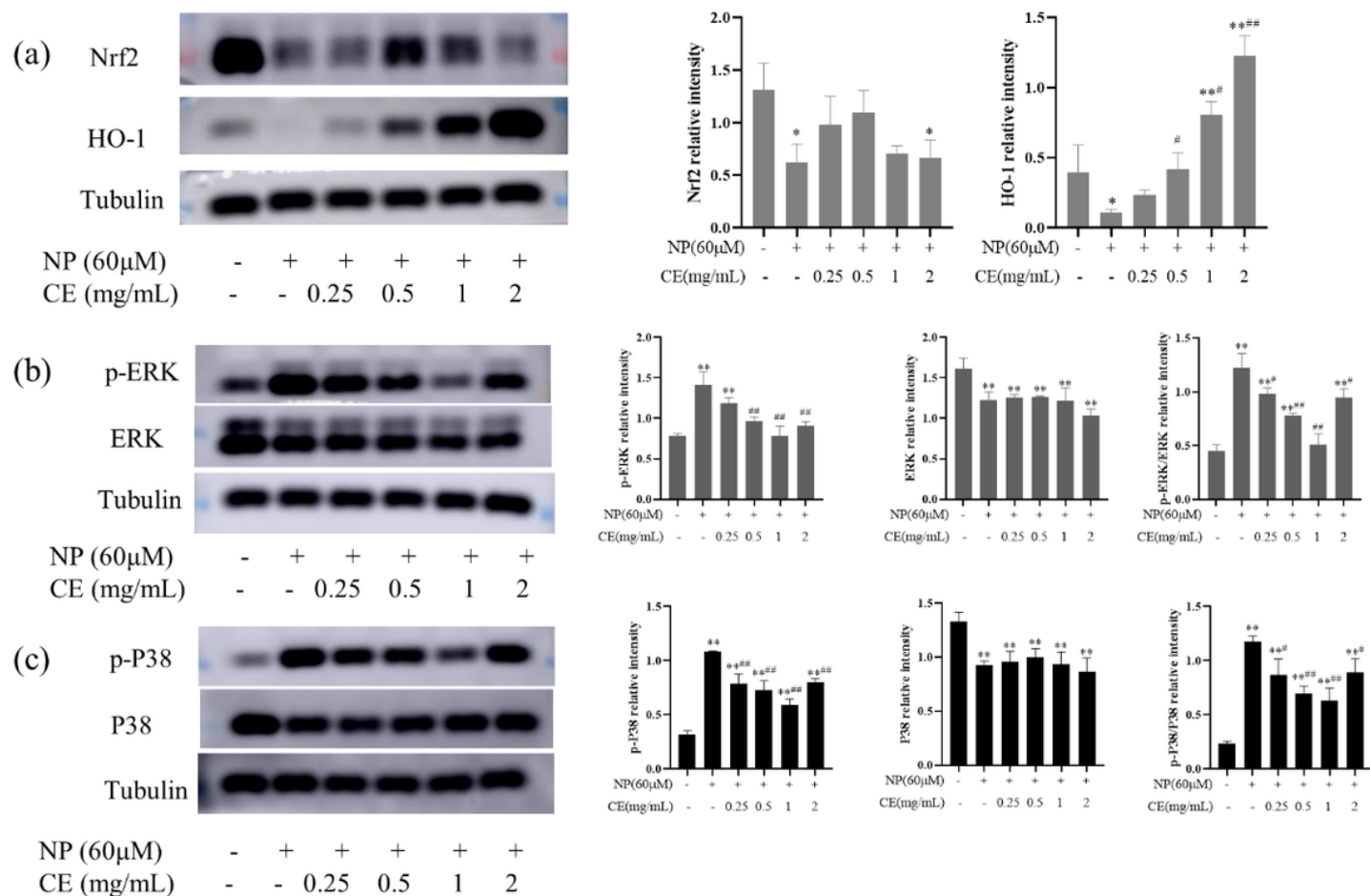


Figure 5

CE alleviates the cytotoxicity of NP via MAPKs/Nrf2 pathway(a) CE alleviates the cytotoxicity of NP via Nrf2/HO-1 pathway(n=3); (b) (c) CE alleviates the cytotoxicity of NP via ERK1/2 and P38 pathway (n=3). (*: $P<0.05$ compared with the control group; **: $P<0.01$ compared with the control group; #: $P<0.05$ compared with the NP group; ##: $P<0.01$ compared with the NP group.)

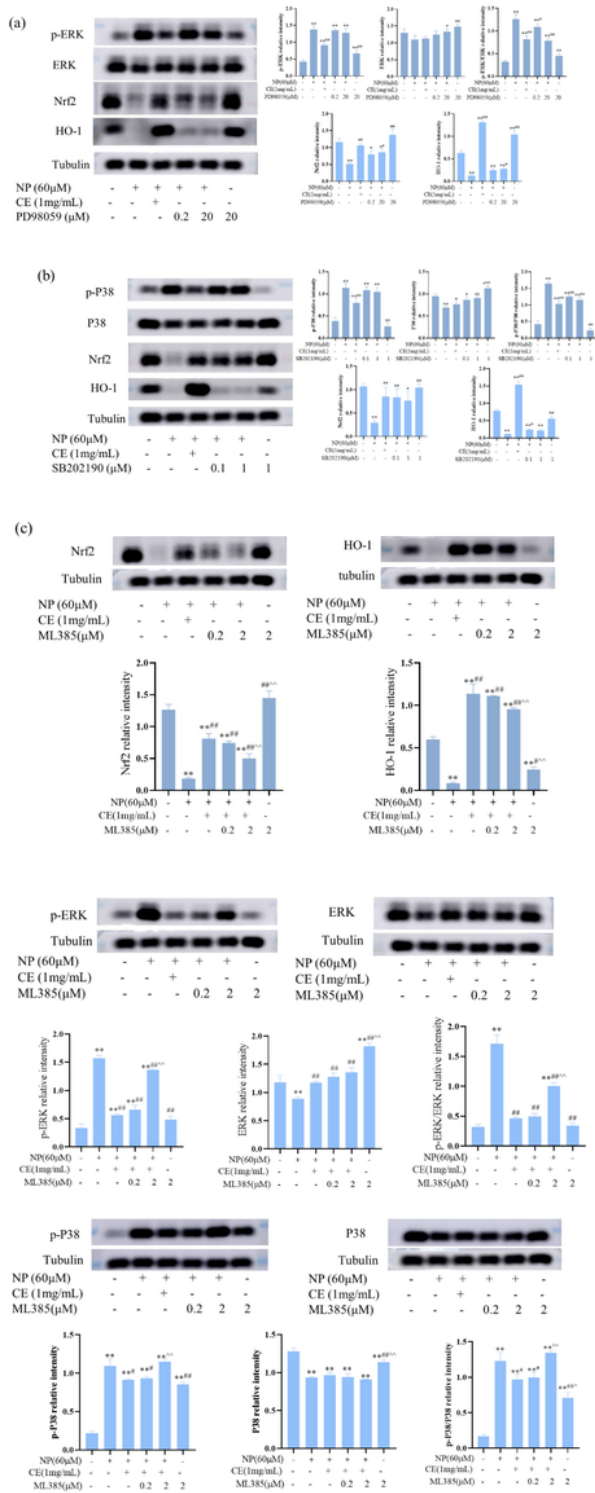


Figure 6

ERK inhibitor (PD98059), P38 inhibitor (SB202190), and Nrf2 inhibitor (ML385) were used for verifying CE alleviates the cytotoxicity of NP mainly depending on ERK1/2 and P38 to affect Nrf2/HO-1 pathway (a) ERK inhibitor (PD98059) was used to verify CE alleviates the cytotoxicity of NP, mainly depending on the ERK1/2 pathway. (n=3); (b) P38 inhibitor (SB202190) was used for verifying CE alleviates the cytotoxicity of NP mainly depending on the P38 pathway; (c) Nrf2 inhibitor (ML385) was used for verifying CE

alleviates the cytotoxicity of NP mainly depend on Nrf2/HO-1 pathway. (*: $P<0.05$ compared with the control group; **: $P<0.01$ compared with the control group; #: $P<0.05$ compared with the NP group; ##: $P<0.01$ compared with the NP group; ^: $P<0.05$ compared with the NP and CE Co-cultivation group; ^^: $P<0.01$ compared with the NP and CE Co-cultivation group)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphabstract.docx](#)
- [Supplementarymaterialwesternblot.pdf](#)
- [Supplementarymaterialwesternblot.zip](#)